ANIMAL GENE THERAPY

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to DNA sequences, expression cassettes and DNA constructs for use in therapy, specifically in gene therapy for the treatment of infectious diseases such as mastitis. Also included are pharmaceutical and veterinary compositions containing the constructs, and cells which have been transformed with the DNA and which are suitable for implantation into a host mammal.

(b) Description of Prior Art

At the highest level, transgenic animals are the principal way to confer transmissible resistance to diseases in animals. Only few years after the first successful gene transfer into mice the new technique was used in farm animals. Several genetic treats have been targeted for the application of transgenesis in domestic animals, but one of those important aspects is the improvement of animal health and disease resistance by gene transfer means. Transient as well as stable genetic improvement leading to disease resistance and treatment achieved by recently developed techniques in molecular biology may contribute considerably to reduce the problem of diseases.

Resistance to infections in animals elicited at various levels. Constitutional and phagocytic mechanisms (innate immunity) serve as a first line of defense. If these are ineffective the infected organism can respond by means of specific (acquired) immunity. Thus, candidates for gene therapy applications include all genes known to modulate non-specific and specific host defense mechanisms, i.e. cytokines, major histocompatibility complex (MHC) proteins, T-cell receptors (TCR) and proteins conferring specific dis-

ease resistance. Increased protection against pathogens can be conferred also by other strategies such as "intracellular immunization", genetic immunization, antisense sequences as anti-pathogenic agents and disruption of disease susceptibility genes.

Gene modulating Immune Responses

Cytokine orchestrate immune responses through their role as soluble mediators of cell communication. Initially identified to direct viability, proliferation, differentiation and homing of leukocytes, they were also found to regulate the production of function or one another. In addition, cytokines interact with, and are produced by cells other than leukocytes, thus providing a means of communication between the immune system and other tissues and organs. Cytokines represent a rapidly growing number of regulatory peptide factors including growth factors, interleukins, chemokines, colony-stimulating factors and interferons. Their functions are mediated through binding to cell surface receptors on their target cells. Cytokines have been shown to contribute directly to the development of pathology during infectious diseases and tumorigenesis. Different cytokines have been reported to both positively and negatively influence host defense mechanisms.

Interferon (IFNs) are a well characterized class of cytokines eliciting antiviral and antiproliferative activity as well as modulating cell growth, differentiation and immune responses. As well as their more characterized antiviral activity, IFNs are instrumental in counteracting non-viral pathogens mostly through their effects on macrophage activation. The proteins known to be involved in the antiviral and bactericidal actions of interferon and their inhibitory mechanisms are numerous. The potency of IFNs to posi-

tively influence host susceptibility to viral infections was tested in transgenic mice and cell lines. Transgenic organisms overexpressing IFN-β gene constructs were shown to exhibit enhanced viral resistance.

Recent progress in the understanding of signal transduction pathways and transcription factors activated by IFNs and a variety of other cytokines promises to open up new therapeutic approaches as well as novel strategies of gene transfer treatments aiming at the improvement of the immune response; i.e. the transfer of cytokine encoding genes *per se* of distinct "cytokine-specific" signaling components. Constitutive expression of an interferon-stimulated gene factor (ISGF2) also termed interferon regulatory factor (IRF-1) transgenes has been reported to result in IFN-independent activation of various IFN-inducible genes and enhanced resistance to viral infection.

Specific Disease Resistance Genes

Other improvement which can be brought to animals by local gene transfer is specific disease resistance. A well examined specific disease resistance gene is the Mx1 gene product of certain mouse strains. The mouse Mx1 protein belongs to a family of polypeptides with GTPase activity synthesized in IFN-treated vertebrate cells. Some Mx proteins have been shown to block the multiplication of certain negative-stranded RNA viruses, as for example Influenza virus , VSV, rhado virus and Thogoto virus. Synthesis of mouse Mx1 protein in various cell lines and transgenic mice demonstrated that it is both necessary and sufficient to promote resistance to influenza A viruses in previously susceptible cells and animals. The cloning and functional characterization of this specific disease resistance gene enabled a gene transfer program to study

whether Mx1 transgenic pigs would show reduced susceptibility to influenza infections.

Natural resistance of certain inbred mouse strains to infection with antigenetically unrelated microorganisms such as *Mycobacteria, Salmonellae* and *Leishmania* is controlled by a dominant locus on chromosome 1 called *Bcg, Lsh* or *Ity* respectively. The locus affects the capacity of the host to restrict proliferation of these infectious pathogens during the non-specific macrophage-dependent phase of infection. A positional cloning approach resulted in the isolation of a candidate *Bcg* gene designated *Nramp*. The reduction of susceptibility to *Salmonella* infections by transgenesis or gene therapy (*in vivo* or *ex vivo*) means is of great value for animal production, especially poultry. Large difference in resistance to *Salmonella* in chicken inbred lines have been observed. Furthermore, natural resistance or susceptibility to infection with *Mycobacteria* in humans and *Brucella* in cattle has been shown to be under genetic control similar to that observed in inbred mice and governed by *Bcg*. Chronic infection of cattle with *Brucella abortus* causes the spontaneous abortion of fetal calves, threatening the economic well-being of the dairy and beef industries.

Genetic resistance to certain retroviruses has been observed as a polymorphic trait in several experimental species. One of the identified loci in mice, *Fv-4*, resembles the 3' half of a murine leukemia virus extending from the end of the *pol* gene through a complete *env* gene. Expression of *Fv-4* encoding only the viral envelope protein in transgenic mice conferred resistance to infection with ecotropic retroviruses. The mechanism of *Fv-4* resistance is thought to be related to the phenomenon of viral interference, i.e. competition of the synthesized envelope protein with

exogenous virus for the virus receptor. Similar mechanisms are used in antiviral strategies known as "intracellular immunization".

Expression of a transgene encoding an immunoglobulin specific for a common pathogen can provide immunity for that pathogen. As shown by many investigations, cloned genes coding for monoclonal antibodies can be expressed in large amounts in genetically manipulated mice. These mice produce antibodies against specific antigens without prior contact or immunization.

Intracellular Immunization

The concept of "intracellular immunization" essentially involves overexpression in the host of an aberrant form (dominant-negative mutant) of a viral protein that is able to interfere strongly with the replication of the wild type virus. Elegant studies in cultured cells resulting in acquired resistance to various viruses include strategies preventing virus attachment to the target cells, blocking the formation of virus-host transcription complexes, expressing dominant-negative viral trans-activators or interfering with the assembly of infectious viral particles.

Endogenous mouse mammary tumor virus (MMTV) proviruses have been found to co-segregate genetically with loci termed self-superantigens identical to a protein encoded in the long terminal repeat of MMTV. Genetically manipulated mice expressing high levels of this self-superantigen were shown to be protected from viral infection by deletion of a specific class of T-cells which is the target for infection.

The definition of "intracellular immunization" is also applied for antiviral strategies described in different connections such as expression of specific

resistance genes, antisense RNAs or other antiviral components.

Recently, an "intracellular immunization" approach carried out in farm animals was reported. Transgenic sheep were produced and were shown expressing the visna virus envelope (*env*) gene. The visna virus belongs to a subfamily of ovine retroviruses that cause encephalitis, pneumonia and arthritis in sheep. The env glycoprotein is responsible for the binding of this virus to host cells. The target cell for visna virus replication in infected sheep is the macrophage. The expression of env protein on the cell surface of visna-infected cells induces immune responses to the virus. Expression of a gene construct consisting of the visna U3 enhancer region fused to the *env* gene in transgenic sheep had no obvious deleterious effect. Thus, the genetically manipulated sheep lines provide an evidence for the potential of a retroviral env glycoprotein to prevent infection and/or to modulate disease in its natural host after virus challenge.

Antisense RNA

The use of antisense RNA to inhibit RNA function within cells or whole organisms has provided a valuable molecular biological method. Antisense RNA functions by binding in a highly specific manner to complementary sequences, thereby blocking the ability of the bound RNA to be processed and/or translated. Antisense sequences are considered an attractive alternative to conventional drugs in the therapy of microbial infections, cancer, autoimmune diseases and other malfunctions. Gene transfer experiments with antisense constructs have been carried out in mice and rabbits. Genetically manipulated mice expressing antisense RNA targeted to the retroviral packaging sequences of Molony murine leukemia virus did not develop leukemia

following challenge with infectious viruses. Transgenic rabbits expressing an antisense construct complementary to adenovirus h5 RNA were produced. Primary cells from these rabbits were found to be 90-98% more resistant to adenovirus infection than cells from control animals.

The use of antisense RNAs as anti-parasithogenic agents can be developed to result not only in RNA-RNA hybrids but catallytically cleave a phosphodiester bound in the target RNA strand. Four structural motifs (hammerhead and hairpin first identified in plant RNA pathogens, the delta motif found in human hepatitis delta virus and a less well characterized motif from *Neuspora*) have thus far been described as intermediates in these self-cleavage reactions. By flanking the hammerhead motif of this ribozyme family with antisense sequences, the cleavage of specific target RNAs has been demonstrated. A large number of substrate molecules can be processed by the catalytic RNA because the ribozyme *per se* is not consumed during the cleavage reaction. Bovine leukemia virus (BLV), a retrovirus , causes persistent lymphocytosis and B-lymphocyte lymphoma in cattle and sheep. A hammerhead ribozyme flanked by antisense sequences directed against regulatory proteins of BLV was shown to inhibit BLV expression in persistently infected cells. This demonstrates the possibility of generating localized (*in vivo* or *ex vivo*) or generalized (transgenic animals) gene therapies that will be resistant to BLV-induced diseases.

Somatic Gene Transfer Approaches

Somatic gene transfer into farm animals will become more significant. *Ex vivo* and more recently *in vivo* gene therapy has been applied for several genetic diseases in human. Current therapies developed for

more than 10 gene human disorders, such as failing genes coding normally for the adenosine deaminase, LDL receptor, glucocerebrosidase, blood clotting factor VIII, phenylalanine hydroxydase, dystrophin and others. The efficiency of the gene therapy approach has no more to be proved.

Novel methods for gene transfer into somatic cells promise to be highly efficient. These include viral vectors for delivering gene constructs and non-viral technologies, such as micro-bombarding or injection of DNA particles or solutions into tissues or blood vessels. Although most efforts are directed primarily towards the possibility of treating human diseases, some applications of somatic gene transfer could be of great value in veterinary medicine. It makes direct "genetic immunization" and other methods of immunomodulation possible. "Genetic immunization", i.e. application of DNA constructs encoding immunogens, has at least two powerful uses. One is to simplify the procedure and to shorten the time required to produce antibodies to particular proteins by eliminating the steps for protein purification. it would be more rapid again to introduce a gene encoding directly a neutralizing or bacteriocid antibody in the organism. The second is the genetic vaccination of animals against infections by producing foreign antisense encoded by appropriate gene construct.

The somatic gene transfer approach can now be applied also both to cure and prevent an infectious diseases by releasing in the organ, or in the organism, a protein which is lethal and absolutely specific for the targeted microorganism and without any affinity or effect for the animal.

Such proteins or peptides having a high and specific antimicrobial activity are divided into two

families, one including the bacteriocins and the other the lanthionines, also called lantibiotics. The application of biotechnology to animal treatment, particularly farm animals, is opening up new avenues of prevention and control that will have important implications. The bacteriocins consist of enzymes and other bactericidal proteins. They act as catalysts and are very specific to a single chemical reaction. Bacteriocins kill targeted organisms rapidly by lysing the cell wall, and they do not require that the organism undergo cell division. They are produced naturally by bacteria as a means of population control. These proteins are larger molecules than antibiotics and are expected to persist in the treated organ longer. One of these well known bacteriocins is lysostaphin, which is produced by *Staphylococcus simulans* biovar *staphylolyticus*. Unlike antibiotics, the rapid action of bacteriocins reduces the likelihood of an induced resistance in target and non-target organisms. For example, current research conducted so far seems to indicate that bacteriocins used for mastitis treatment are non-toxic to other organisms.

Lantibiotics are peptide-derived antibiotics with high antimicrobial activity against several pathogenic bacteria. The ribosomal origin of lantibiotics was first shown by the isolation of the structural gene, *epi*A, for epidermin, a lantibiotic produced by *Staphylococcus epidermidis*. The general structure of lantibiotic genes is the same in all lantibiotics described so far. The primary transcript of linear lantibiotics is a prepeptide which consists of an N-terminal leader sequence that is followed by the C-terminal propeptide from which the lantibiotic is matured and a characteristic proteolytic processing site with proline at position -2. Nisin, produced by

several Lactococcus lactis strains, is a prominent member of the group of lanthionines.

Other bacteriocins and lanthionines are ambicins, defensins, cecropins, thionins, mellitins, magainins, attacines, diphterins, saponins, cacrutins, xenopins, subtilins, epidermins, pep5, lacticin 481, ancovenins, duramycins, gallidermins, cinnamycins, andropins and mastoparans.

Another new class of molecule complexes which can be secreted by the transgene, i.e. the genetic construct used for a gene therapy application is the immunoadhesins. The therapeutic potential of antibodies has long been recognized Human antibodies should be minimally immunogenic to the patient; they should therefore be safe for chronic or repeated use. However, it can be difficult to generate useful human antibodies for several reasons: it is ethically impossible to immunize human beings for experimental purposes, thus the available human antibodies are limited to the products of inadvertent immunization or vaccination. Furthermore, there have been technical difficulties in the immortalization of human cell lines. Perhaps the most refractory technical problem is that many applications require antibodies to human antigens; since human antibodies with the desired specificity.

Several potential approaches exist to circumventing these problems. One approach is to engineer the desired specificity of binding into human antibody variable(V) regions. This can be done by deriving the complementary determining regions either from mouse antibodies, or from *in vitro* recombination combined with selection (e.g. combinatorial libraries and phage display technology). An alternative approach, which sometimes has advantages, is to create an antibody-like molecule by combining a binding site, derived from a

human protein such as a cell-surface receptor or cell-adhesion molecule, with antibody constant domains. Such molecules are known as immunoadhesins.

Immunoadhesins can possess many of the desired chemical and biological properties of antibodies. Examples exist of immunoadhesins that can bind to Fc receptors, mediate antibody-dependent cellular cytotoxicity, and show active transport across the primate placenta. Since the immunoadhesin is constructed from a receptor sequence linked to an appropriate hinge and Fc sequence, the binding specificity of interest can be achieved using entirely human components. Another potential foreign sequence is that in the joining region.

One of well studied immunoadhesins is CD4-IgG which as been found entirely non-immunogenic in human clinical trials. A second candidate for clinical use is a rumor necrosis factor receptor immunoadhesin (THFR-IgG); this molecule is particularly interesting, since the soluble receptor itself is found naturally in the body and has been considered as a possible therapeutic. While soluble receptors are valid clinical candidates, the IgG fusion form may well confer advantages such as longer half-life and improved avidity and affinity. Some receptors or immunoinducers that have been joined to the Fc part of IgG to form immunoadhesins are reported in the literature: T cell receptor, CD4, l-selectin, CD44, CD28, B7, CTLA-4, CD22;, TNF receptor, NP receptor, IgE receptor, INF-γ receptor. These immunoadhesins should be useful in antigen recognition, reception to HIV, lymphocyte adhesion, receptor for hyluronidase, interaction B and T lymphocytes, inflammation, septic shock, homeostasis and allergy.

In animals, the advent of molecular biology techniques allow to create an immunoadhesin which could

has two specific activities. For example, in the goal to eliminate a contamination with *Staphylococcus aureus* it can be possible to have an immunoadhesin composed of a lytic enzyme, like the lysostaphin, linked to the Fc part of the human IgG which has a high affinity for the protein A at the surface of the bacteria. Once the Fc is linked to the protein A on *Staphylococcus aureus*, the lytic part, the lysostaphin, can lyse the bacteria.

The gene therapy treatments can be applied in such a way that the gene included in the constructs transferred could be coding for an immunomodulator, such as interleukins, chemokines, interferons, leukotriens, and certain growth factors. As explained before, the immunomodulators can makes the animal more resistant to several microorganisms.

SUMMARY OF THE INVENTION

The present invention relates to the animal gene therapy. Animal gene therapy means an approach by which a DNA construct involving an inducible or constitutive promoter linked to a gene coding for a curative or protective protein or antisense RNA or peptide which acts against infectious or potentially infectious microorganisms responsible of the diseases. Disclosed is a method for expressing a protein or antisense RNA or peptide which directly or indirectly has a therapeutic or prophylactic effects against infectious microorganisms in an animals. The invention is useful for producing a heterologous or homologous protein or antisense RNA or peptide which is tethered to a specific tissue or organ and which can act on a microorganisms infecting the animal. The method involves inducing a liquid complex including a genetic construct into a determined tissue of the animal. If desired, the infused genetic construct can be treated with a

polycationic compound and/or a lipid to improve the efficiency with which it is taken up by secretory cells of the animals.

The most costly infectious disease in animals is mastitis caused by the infection of the mammary gland. Among others, this invention relates to a method of treating mastitis. More particularly, this invention relates to the use of DNA constructs designed to be transcribed in a therapeutic protein after insertion into the mammary gland of both lactating or non-lactating animals.

Bovine, caprine, ovine and porcine mastitis remain some of the most costly diseases in animal agriculture. Mastitis represents a significant economic loss to the diary industry, approximately 70 to 80 percent of which can be attributed to a decrease in milk production. Many infective agents have been implicated as causes of mastitis and these are dealt with separately as specific entities in cows, sheep, goats and pigs.

Despite significant progress in mastitis control due to widespread adoption of post-milking teat antiseptisis, many herds continue to be plagued by this disease. A variety of different procedures have been described and used to cure mastitis caused by bacteria and yeast. These procedures include the systemic immunization of the infected animals with whole or partial protein extracts of the infective agents in order to stimulate the immune response of the treated animal to these agents. Antibodies generally produced in this way act against a membrane protein, a binding protein or a toxin secreted by the microorganisms. Hence these antibodies act as anti-adhesive, anti-toxin, neutralizing or opsonic molecules (Nordhaug et al., 1994, *J Dairy Sci.*, **77**:1267 & 1276). Nevertheless, the blood-

milk barrier prevents all but a very small proportion of circulation IgG antibodies from reaching mammary secretion during lactation.

Other procedures have been carried out in order to stimulate the diapedesis and phagocytosis of contaminating agents by leukocytes, more particularly polymorphonuclear neutrophils and macrophages. The stimulating molecules, which have been administered by intramammary injection, are cytokines, interleukin-1β, interleukin 2, interferon-γ, tumor necrosis factor-α.

The most widely used procedure to cure infectious diseases is administration of antibiotics. However, this approach inflicts a lot of side effects to the animal and particularly in the case of dairy animals, the milk must be discarded during the treatment period. Unfortunately, all current procedures are very short-lasting and consequently relatively inefficient. For example, none of the gram positive bacteria are entirely eliminated from the udder after treatments with antibiotics.

For these reasons a gene therapy procedure is desired that allows a gene to be integrated into a targeted tissue, such as mammary gland, and provides for the elimination, by genetic therapy, of the contaminating microorganisms. In addition, gene therapy of the mastitic gland eliminates all the side effects of other procedures, enabling also an inserted gene to synthesize inductively or constitutively in a permanent manner an effective amount of its therapeutic protein, peptide or RNA antisense product. Therefore, this invention allows a much more specific and effective system of infectious diseases treatment than is currently possible.

Additional objects, features, and advantages of the invention will become apparent to those skilled in

the art upon consideration of the following detailed description of preferred embodiments exemplifying the best mode of the invention as presently perceived.

The present invention provides a recombinant DNA which comprises a nucleotide sequence which encodes a protein or polypeptide which is useful in the prophylaxis or treatment of mastitis, and at least one regulatory control element which allows for expression of said nucleotide sequence in a mammary gland.

Suitable regulatory control elements include transcription and translation regulatory sequences. Transcription and translation regulatory sequences are those DNA sequences necessary for efficient expression of the product. In general, such regulatory elements can be operably linked to any nucleotide sequence to control the expression of the sequence, the entire unit being referred to as the "expression cassette". Hence the invention further provides an expression cassette containing the above-mentioned recombinant DNA.

An expression cassette will typically contain, in addition to the coding nucleotide sequence, a promoter region, a translation initiation site and a translation termination sequence.

Unique endonuclease restriction sites may also be included at the end of an expression cassette to allow the cassette to be easily inserted or removed when creating DNA constructs for use in transformations as is known in the art.

In particular the invention provides a DNA construct designed to express a protein or polypeptide which is useful in the prophylaxis or treatment of infectious diseases after insertion into the targetted tissues. Suitably the DNA construct comprises an inducible or constitutive promoter which is linked to a coding nucleotide sequence or gene and thereby

expresses a therapeutic or protective protein which acts against infectious or potentially infectious microorganisms responsible for the diseases of animals.

For example, such DNA constructs can be administered to both lactating or non-lactating animals for the prophylaxis or treatment or mastitis. Hence the invention further provides a method for the prophylaxis or treatment of mastitis which comprises transformation of mammary gland tissue with a DNA construct as described above.

The present applicants have found that expression of proteins in mammary glands over an extended time period is possible and that a gene therapy approach to the problem of mastitis is feasible. Integration of a gene which encodes a therapeutic protein or polypeptide into mammary gland tissue would allow, for example, for the elimination of infective microorganisms by genetic therapy. In addition, gene therapy of the mastitis gland eliminates all side effects of other procedures, also enabling an inserted gene to synthesize permanently and inductively or constitutively an effective amount of its therapeutic protein product. A gene therapy approach would be a much more specific and effective system of mastitis treatment than is currently available.

Transformation of mammary gland tissue generally requires that the DNA be physically placed within the host gland. Current transformation procedures use a variety of techniques to introduce naked DNA into a cell and these can be used to transform a mammary gland. For example, the DNA can be injected directly into glands through the use of syringe. Alternatively, high velocity ballistics can be used to propel small DNA associated particles into the gland through an udder's skin incision.

The DNA can also be introduced into a mammary gland by insertion of other entities which contain DNA. These entities include minicells, cells (e.g. fibroblasts, adipocytes, epithelial cells, myoepithelial cells, mammary carcinoma cells, kidney cells), liposomes (e.g. natural or synthetic lipid vehicles, cationic liposomes) or other fusible lipid-surfaced bodies. The entities are transformed *in vitro* prior to insertion using the above-described DNA constructs.

Thus the invention also provides a cell which has been transformed using a DNA construct as described above. Examples of such cells include Mac-T cells. Genetically transformed cells of this type are suitable for reimplantation into a mammary gland to produce the desired proteins or polypeptides.

Furthermore the invention provides a liposome which incorporates the above-described DNA construct.

Introduction of the naked or complexed DNA constructs into the mammary gland can be performed by direct injection through a skin incision of the udder or through the teat canal.

Where appropriate, the DNA construct is administered in the form of a pharmaceutically or veterinary acceptable composition in combination with a suitable carrier or diluent. Suitable carriers are liquid carriers such as water, salts buffered saline or any other physiological solutions. These compositions form a further aspect of the invention.

The protein or polypeptides produced should be effective prophylaxis or treatment of mastitis. Such proteins or polypeptides include mucolytic proteins such as enzymes, antibiotics, antibodies, cytokines, tumor necrosis factors as well as proteins which can induce an immune response to infective or potentially

infective agents and those which activate polymorphonuclear neutrophils, or macrophages.

In a preferred embodiment, the invention provides a recombinant DNA sequence which comprises a nucleotide sequence which encodes a lytic protein or antibody under the control of a mammary gland specific promoter, or any ubiquitous or inducible non mammary promoter.

The invention is particularly applicable for the treatment of farm animals: bovine, caprine, ovine, and porcine, but can concern also lower mammals or lower milk producers: rabbit, camel and bison. The invention can also be used in humans to eliminate particularly most *Staphylococci*.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates examples of DNA constructs in accordance with the present invention; and

Fig. 2 illustrates the rate of synthesis of human growth hormone in milk's sheep after injection of cationic liposome-DNA complex into the mammary gland.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with one preferred embodiment of the present invention, animal gene therapy of infectious diseases consists in transfecting a targeted tissue with DNA sequences designed to produce molecules which will be relargued into the organ or the organism, this would than protect the animal against the infecting or potentially infecting microbial agents.

In accordance with another embodiment of the present invention, mastitis gene therapy of mammals consists of transfecting the mammary glands with DNA sequences designed to produce molecules which will be relargued into the udder, this would than protect the

animal against the infecting or potentially infecting microbial agents.

The targeted tissue can also be transformed with other DNA sequences such as gene transcription and translation regulatory sequences. Transcription and translation regulatory sequences are those DNA sequences necessary for efficient expression of the gene product. In general such regulatory elements can be operably linked to any gene to control the gene's expression, the entire unit being referred to as the "expression cassette". An expression cassette will typically contain, in addition to the coding sequence, a promoter region, a translation initiation site and a translation termination sequence. Unique endonuclease restriction sites may also be included at the ends of an expression cassette to allow the cassette to be easily inserted or removed when creation DNA constructs.

The expression of a gene is primarily directed by its own promoter, although other DNA regulatory elements are necessary for efficient expression of a gene product. Promoter sequence elements include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs (bp) upstream of the transcription start site. In most instances the TATA box is required for accurate transcription initiation. By convention, the transcription start site is designated +1. Sequences expending in the 5' (upstream) direction are given negative numbers and sequences extending in the 3' (downstream) direction are given positive numbers.

Promoters can be either constitutive or inducible. A constitutive promoter controls transcription of a gene at a constant rate during the life of a cell, whereas an inducible promoter's activity fluctuates as determined by the presence (or absence) of a specific inducer. The regulatory elements of an inducible pro-

moter are usually located further upstream of the transcription start site than the TATA box. Ideally, for experimental purposes, an inducible promoter should possess each of the following properties: a low to non-existent basal level of expression in the absence of inducer, a high level of expression in the presence of inducer, and an induction scheme that does not otherwise alter the physiology of the cells. The basal transcription activity of all promoters can be increased by the presence of "enhancer" sequences. Although the mechanism is unclear, certain defined enhancer regulatory sequences are known, to those familiar with the art, to increase a promoter's transcription rate when the sequence is brought in proximity to the promoter.

Constitutive promoters can activate the transcription of its linked gene in a tissue specific manner, such as those naturally actives in the epithelial cells of a mammary gland. For example, strong constitutive promoters are those controlling the expression of caseins, lactoglobulins, lactoferrin, lactalbumin, lysosymes, whey acidic proteins (WAP) coding genes in mammary glands. Preferentially, the promoters originates from domestic animals, bovine, caprine, ovine or porcine species. Alternatively, specific mammary gland promoters can originates from smaller animals, lagomorphes, rodents, felines or canines. Other constitutive promoters regulating expression of the cytoplasmic β-actin or ubiquitin genes can be used.

Viral or retroviral promoters can be used also, like Cytomegalovirus (CMV), Simian virus 40 (SV40) or mouse mammary tumor virus (MMTV, which is additionally inducible).

Inducible promoters include any promoter capable of increasing the amount of gene product produced,

by a given gene, in response to exposure to an inducer. Inducible promoters are known to those familiar with the art and a variety exist that could conceivably be used to drive expression of the protective or curative molecule's gene.

Two preferred inducible promoters are the heat shock promoter (HST) and the glucocorticoid system. Promoters regulated by heat shock, such as the promoter normally associated with the gene encoding the 70 kDa heat shock protein, can increase expression several-fold after exposure to elevated temperatures. The heat shock promoter could be used as an environmentally inducible promoter for controlling transcription of the protective or curative molecule's gene. The glucocorticoid system also functions well in triggering the expression of genes including protective or curative molecule's gene. The system consists of a gene encoding glucocorticoid receptor protein (GR) which in the presence of a steroid hormone forms a complex with the hormones. This complex then binds to a short nucleotide sequence (26 bp) named the glucocorticoid response element (GRE), and this binding activates the expression of linked genes. The glucocorticoid system can be included in the DNA transformation construct as a means to induce protective or curative molecule's expression. Once the constructs have been inserted the systemic steroid hormone or glucocorticoid will associate with the constitutively produced GR protein to bind to the GRE elements, thus stimulating expression of the protective or curative molecule's genes (e.g. antibodies or enzymes).

Presumably the targeted tissue will allow the inserted gene (naked, liposome, cell-enclosed or coated solid particle) to produce its protein product in an amount sufficient to produce the desired effect. The

inserted gene's products must cure or protect the organ or the organism in which it is expressed against infectious or potentially infectious microorganisms responsible or potentially responsible of the disease.

The transformation of an animal tissue requires that the DNA be physically placed within the host animal. Current transformation procedures utilize a variety of techniques to introduce naked DNA into a cell, that can be used to transformed a targeted tissue. In one form of transformation, the DNA is injected directly into the tissue though the use of syringe. Alternatively, high velocity ballistics can be used to propel small DNA associated particles into the tissue through a skin's incision. In other forms, the DNA can also be introduced into a targeted tissue by insertion of other entities which contain DNA. These entities include minicells, cells (e.g. fibroblasts, adipocytes, Mac-T cells, myoepithelial cells, mammary carcinoma cells, kidney cells, liver cells, lung cells, lymphocytes, leukocytes), liposomes (e.g. natural or synthetic lipid vehicles, cationic liposomes) or other fusible lipid-surfaced bodies.

The invention is concerned when a neutralizing, lytic or opsonic molecules are synthesized from the gene used for the infectious disease's gene therapy. Preferentially, in the case of the mastitis, the gene coding for a mucolytic protein (e.g. bacteriocins and lanthionins) can be used to eliminates the Gram positive bacteria (mostly *cocci*). The gene products can serve as an immunomodulator and to induce an immunologic response, the activation of polymorphonuclear neutrophils, or macrophages for example. The product can be a cytosin or other immunomodulator. Alternatively, the genes can be used for in-situ synthesis of the following therapeutic polypeptides:

1. Enzymes or mucolytic proteins, such as lysostaphin and mucolysins;
2. Antibodies, such as anti-hemolysins, anti-leucocidin, anti-protein A, anti-collagen, anti-fibronectin binding protein, anti-laminim, anti-α-toxin and anti-β-toxin antibodies; opsonic antibodies and antibodies raised against cell fusion viral protein;
3. Cytokines, interleukines, chemokines, growth factors;
4. Interferons;
5. Tumor necrosis factors; and
6. Immunoadhesins or immunotoxins.

While antibiotics are not very suitable, it can be alternatively used with inducible promoters.

Microorganisms which can be responsible of the mastitis and be eliminated by the gene therapy approach are:

In cattle *Streptococcus agalactiae, Str. ube, Str. zooepidemicus, Str. dysgalactiae, Str. faecalis* and *Str. pneumoniae, Straphylococcus aureus, Escherichia coli, Klebsiella spp., Corynebacterium pyogenes, Cor. bovis, Mycobacterium tuberculosis, Mycobacterium spp., Bacillus cereus, Pasteurella multocida, Pseudomonas pyocyaneus, Sphaerophorus necrophorus, Serratia marcescens, Mycoplasma spp., Nocardia spp.*, a fungus *Trichosoporon spp.*, yeasts *Candida sp., Cryptococcus neoformans, Saccharomyces*, and *Torulopsis spp.*.

In sheep: *Pasteurella haemolytica, Staph. Aureus, ActinoBacillus lignieresi, E. coli, Str. uberis* and *Str. agalactiae*, and *Cor. pseudotuberculosis.*

In goats: *Str. agalactiae, Str. dysgalactiae, Str. pyogenes,* and *Staph. aureus.*

In pigs: *Aerobacter aerogenes, E. coli, Klebsiella spp., Pseudomonas aeruginosa, coagulase-positive Staphylococci, Str. agalactiae, Str. dysgalactiae,* and *Str. uberis.*

In horses: *Corynebacterium pseudotuberculosis, Str. zooepidemicus,* and *Str. equi.*

Other microorganisms and diseases which can be eliminated from exotic animals by the method of gene therapy are those causing:

In primate: Poliomylltis, Measles, Mumps, Rubella, DPT, Tetanus.

In canidae: Can. distemper, Can. adenovirus, Can. parvovirus, Can. parainfluenza, Rabies, Leptospire bacterin.

In felidae: Fel. panleukopenia, Fel. rhinotracheitis, Fel. caliciviruses, Rabies.

In Artiodactyla: BVD, 8-way Clos. bacterin, 5-way Lepto. bacterin, Parainfluenza 3, Prions, Scatters.

Examples of infectious diseases which could be cured or prevented by the application of gene therapy are: anemia, arthritis, rhinotracheitis, bronchitis, bulbar paralysis, bursal diseases, hepatitis, cloacitis, coryza, enterohepatitis, hemopoietic necrosis, jaundice, keratoconjunctivitis, laryngotracheitis, myxomatosis, necrotic hepatitis, ophthalmia, pancreatic necrosis, pododernatitis, polyarthritis, pustular balanoposthitis, vulvovaginitis, serositis, sinusitis, stomatitis, synovitis, thromboembolic meningitis, and tracheobronchitis.

The present invention concerns a gene therapy approach with both curative and prophylactic activities on causing diseases infectious microorganisms. The

invention concerns in particular DNA sequences, expression vectors, DNA carriers (lyposome, solid particles) and cells allowing to make use of the process.

The invention concerns equally the cells (e.g. Mac-T, lung, kidney, muscle cells) genetically transformed *in vitro* with the gene of interest and reimplanted into the originating tissues to produce the curative or prophylactic proteins, peptide or antisense RNA against microorganisms responsible or potentially responsible of the diseases.

The invention concerns more particularly domestic animals: bovine, caprine, ovine, porcine, feline, canine and birds, but can concerns also more exotic animals such as rabbit, camel and bison.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Long-term persistence of plasmid DNA and foreign expression in sheep mammary glands

Mammary-gland promoters have been used in transgenic animals to limit transgene expression to the mammary gland. Gene therapy techniques to target just one organ for introduction of a foreign gene have also been demonstrated. Most efforts toward postnatal gene therapy have relied on new genetic information into tissues: target cells are removed from the body, infected with viral vectors carrying the new genetic information, and then reimplanted into the body. For some applications, direct introduction of genes into tissues *in vivo*, with or without the use of viral vectors, would be useful. Direct *in vivo* gene transfer into postnatal animals has been achieved with formulations of DNA encapsulated in liposomes, DNA entrapped

in proteoliposomes containing viral envelope receptor proteins (Nicolau et al., 1983, *PNAS USA*, **80**:1068), calcium phosphate-coprecipitated DNA (Benvenisty et al., 1986, *PNAS USA*, **83**:9551), and DNA coupled to a polylysine-glycoprotein carrier complex (Wu and Wu, 1988, *J. Biol. Chem.*, **263**:14621). *In vivo* infectivity of cloned viral DNA sequences after direct intrahepatic injection with or without formation of calcium phosphate coprecipitates has also been described (Seeger et al., 2984, *PNAS USA*, **81**:5849). With the use of cationic lipid vesicles (Felgner et al., 1989, *PNAS USA*, **84**:7413), mRNA sequences containing elements that enhance stability can be efficiently translated in tissue culture cells (Malone et al., 1989, *PNAS USA*, **86**:6077) and in *Xenopus laevis* embryos (Malone, 1989, Focus 11:61). It is demonstrated here that injection of pure DNA complexed to cationic liposomes directly into sheep mammary gland results in significant expression of reporter gene within the gland.

Preparation of plasmid-liposome mixture

Plasmid pCR3 (InVitrogen) was used as mammalian expression vector. After PCR amplification, the human growth hormone (hGH) cDNA was inserted into pCR3. This resulted in plasmid construct pCR3. Plasmid-LipofectAMINE™ (BRL) mixture was prepared as described by the manufacturer (GibcoBRL). Briefly, 50 ug of pCR3-hGH suspended in 500 µl sterile phosphate buffered saline (PBS), was mixed to 100 µl of LipofectAMINE™ also previously diluted into 500 µl of PBS, and kept at room temperature at 1 hour.

Infusion of the plasmid-liposome complexes into sheep mammary gland

The circular pCR3-hGH plasmid-LipofectAMINE™ mixture was loaded into a glass syringe. Just after dropping, by using a 20-gauge needle, the DNA-liposome

complex was infused directly through the udder's skin into the mammary parenchyma. One ml was injected into the right quarter of two ewes. The milk of the left glands was used as negative controls.

Analysis of sheep milk

Sheep were milked once daily by hand with the milk kept at -80°C until analyzed. The amount of hGH was measured by immunoassay (Immunocorp) after determining that the milk did not affect the accuracy of the assay. Aliquots (100 ul) of milk samples were analyzed.

RESULTS

hGH synthesized by injecting pCR3-hGH into the mammary gland was detected all along the lactating period, meaning about 60 days, as illustrated in Fig. 2. The concentration of hGH in the sheep's milk was relatively high during the first 5 days. At that time it was of 300 to 400 ng/ml (± 43 ng/ml). hGH concentrations in the milk from the left (control) gland was from 10 to 15 ng/ml for the two sheep everyday of the experiment. No important differences of concentration of hGH in milk samples were found between each ewes.

Conclusion

These results demonstrate that expression from plasmid DNA can persist in a sheep's mammary gland for at least 60 days. The unprecedented ability of plasmid DNA to stably express a foreign gene in a mammary gland throughout the lactating period of a sheep has important implications for gene therapy. The stable expression of circular plasmid DNA suggest that foreign acceleration or by viral transduction should also be stably maintained.

EXAMPLE II

Human growth hormone (hGH) secretion in goats' milk after direct transfer of the hGH gene into the mammary gland

An alternative route of introducing genes into the mammary parenchyma is through expansion of gene therapy techniques. In this study two Gibbon ape leukemia virus (GaLV) pseudotype retroviral vectors were used to transfer reporter genes into a goat's mammary secretory epithelial cells *in vitro* and *in vivo*.

Cells and tissue culture

MDBKs, a bovine kidney cell line and Mac-T cells, a bovine mammary epithelial cell line were used. Retroviral packaging cell lines used (φCre, PA317, and PG13/LNc8) were acquired from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamycin (54 mg/ml) and 10% fetal calf serum, 37°C with 5% CO_2 /95% air.

Establishment of producer cell lines

A construct carrying the JR-gal neo- (Wang et al., 1991, *Cancer Res.*, 51:2642) was transfected into the ecotropic packaging cell line φCre by particle bombardment at 1 µg of DNA per mg of gold beads. Two days after bombardment, the supernatant was removed from these cells and centrifuged, and after the addition of Polybrene at 4 µg/ml, the retroviral solution was used to infect both amphotropic and GaLV pseudotype packaging cell lines. A plasmid carrying the retrovirus vector, MFG-hGH was cotransfected with pSV2neo at a ratio of 50:1 via particle bombardment into PA317s and PG13/LN c8s. Packaging cells producing retrovirus containing the hGH gene were selected by G418 resistance (400 µg/ml).

Virus producing cells

The PG13/LN c8 clones that yielded the highest levels of hGH produced from the target cell lines were chosen for the infusions into a goat's mammary glands. Each clone was passed three times into 200 100 mm-plates. Cellular supernatant was collected over a 3-day period, concentrated, and resuspended in DMDM with Gentamycin.

Induction of cell division and lactation of goats

Two 2-year-old (goats 1 and 2) and two 1-year-old (goats 3 and 4) virgin Saanen-crossbred goats were treated with exogenous steroids i.m. over a 14-day interval to induce mammogenesis and subsequent lactation.

Infusion of viral stocks into a goat's mammary glands

Polybrene was added to concentrated PG13/LN c8 MFG-hGH viral stock at 80 µg/ml and loaded into a syringe. By using a 22-gauge stub adapter, the retroviruses were infused up the right mammary teat on days 3, 5, 7, 9, 11, and 13 of the hormonal regimen for goats 1, 2, and 4 and goat 3 received infusions on days 3, 5, 7, 9, 10, and 13. The amount of viral solution was different for each animal, ranging from 8 to 20 ml, and was determined by the integral capacity of the gland. The left gland served as the intraanimal control and was infused with DMEM containing gentamycin. Retroviral stock used for the infusions was then assayed on several cell lines.

Analysis of goat's milk

Goats were milked twice daily by hand with the morning milk kept at -80°C until analyzed. The amount of hGH was measured by immunoassay after determining that the milk did not affect the accuracy of the assay.

Aliquots (5 µl) of milk samples diluted 1:10 in double distilled water were also analyzed by SDS/PAGE on 14% gels stained with Coomassie blue. The protein concentration of the milk samples was determined by using BCA (Pierce et al., 1977, *Anl. Biochem.*, **81**:478).

RESULTS

Vector production of packaging cell lines

The concentration of hGH in the medium removed from Mac-T and MDBK cells 2 days after infection with retrovirus packaged by PG13/LN c8 clone 6 was 192 and 3.8 ng/ml, respectively. Twenty-eight days after infection, hGH levels from these cells were 119.3 and 4.5 ng/ml, indicating that the provirus LTR was still functioning 4 weeks after infection.

Infusion of viral stocks into the mammary glands of goats

Viral stock infused on day 13 for goats 1 and 2 was found to contain hGH at 224 ng/ml, indication that the PG13/LN c8 packaging cell were also producing hGH.

Analysis of goat milk

Lactation commenced on day 14 of the hormonal regimen, 24 hr after the last viral infusion. Milk appeared normal throughout the lactations. The volume of milk obtained from each udder half was approximately 150 ml on the first day of lactation for goats 1 and 2 but only 10 ml for goat 3, and 35 ml for goat 4. Milk volume produced by each gland for all four goats increased daily. The levels of hGH were determined by immunoassay with unique hGH secretion patterns for each animal. In goat 1, concentration of hGH dropped steadily until day 9 of lactation when it leveled at 3-5 ng/ml, whereas goats had a more precipitous decrease in measured hGH from day 1 to day 2 of lactation, though

the animal's production of hGH stabilized at 2-3 ng/ml around day 10. Milking was stopped on day 15 of lactation for goats 1 and 2. Levels of hGH in the milk of goat 3 dropped dramatically from day 1 to 2 of lactation and then increased from day 8 to day 9 where it remained at 23 ng/ml until day 16 when it began to fall again. Goat 4, in which prostaglandin E2 was infused at the end of the remaining 19-day lactation after a decline on the first 2 days. In addition, goat 4 was still secreting hGH at 5 ng/ml after 28 days. hGH concentrations in the milk from the left (control) gland ranged from 0.0 to 0.6 ng/ml for the four goats at all evaluated times. These numbers are at the detection level of the assay and correlate with ones measured in two other lactating goats that had no exposure to retrovirus. The total production of hGH in the four animals ranged from 0.3 to 2 ug/day.

If the hGH gene had been stably incorporated into the stem-cell population, it would have been expected that the goats would also secrete hGH in a second lactation after the gland had undergone involution. A second lactation was induced in two of the goats, and though goat 1 did not produce hGH, goat 2 began secreting detectable amounts of hGH starting on day 5 from the right (infused) gland and during the subsequent 10 days hGH concentrations varied from 0.4 to 2.3 ng/ml. Milk from the left control gland during this lactation always had no detectable levels of hGH.

SDS/PAGE of goat's milk sampled throughout the period of collection showed no consistent differences in the protein profiles from the retroviral-infused right glands, the control left glands, and a goat not exposed to the retrovirus. Protein concentrations measured by BCA of the milk with hGH were not statistically different from the control milk, thus production

of hGH by the mammary secretory epithelial cells did not appear to affect the normal cellular protein machinery. There was an indication that the milk's proteins in the treated gland were not secreted at maximal concentration on day 1 of lactation.

Conclusion

Applying gene therapy technology and replication-defective retroviral vectors to directly introduce a foreign gene into a ruminant mammary gland has dramatically reduced the time of production of pharmaceuticals in milk, from years to weeks. Although the levels of expression found are low, the methods might find application in the evaluation of different gene constructs as a prelude to production of transgenic animals or in the production of low levels of important proteins for evaluation purposes.

EXAMPLE III

Effect of lysostaphin on *Staphylococcus aureus* infections on the mouse's mammary gland

Lysostaphin is an endopeptidase produced by *Staphylococcus simulans*. It hydrolyzes the pentaglycine links of the peptidoglycan of members of the genus *Staphylococcus* and consequently has little activity against other prokaryotes and none against eukaryotes. The lysostaphin gene has been cloned and expressed successfully in *Escherichia coli* and *Bacillus* species (Heath et al., 1987, *FEMS Microbiology Letters*, **44**:129; Heinrich et al., 1987, *Molecular and General Genetics*, **209**:563; Recsei et al., 1987, *PNAS USA*, **84**:1127). The use of lysostaphin to promote lysis of *Staphylococcus aureus* in a variety of experimental situations is well known but the progress made in cloning and expressing the gene in other hosts raises the possibilities of

producing large quantities of the enzyme relatively inexpensively. This may permit its use *in vivo* in new approaches to the control of staphylococcal mastitis, an economically important disease of lactating ruminants (Bramley et al., 1990, *Res. Vet. Sci.*, 49:120). This experiment shows the use of a mastitis model in the lactating mouse and clearly demonstrates potent antibacterial activity of lysostaphin against *S. aureus in vivo*.

Lysostaphin (Sigma Chem.) was dissolved in skimmed milk (Oxoid) to provide a range of concentrations between 0.1 and 100 µg/ml. Controls without lysostaphin were included. One ml volumes of the controls and lysostaphin dilutions were inoculated with 10^8 colony forming units (cfu) of *S. aureus* M60. This strain produces both α and β toxins and was isolated from a case of bovine mastitis. Lysostaphin concentrations exceeding 2 to 3 ug/ml in milk produced a 2 to 3 $\log_{10}$ reduction in viable *S. aureus*, whereas 10 ug/ml in milk reduced *S. aureus* from a mean of 7.95 $\log_{10}$/ml in the control to 2.0 $\log_{10}$/ml. Consequently a dose of 10 ug of lysostaphin was selected for use *in vivo*. Anaesthetized mice, of strain MF1, were inoculated in the upper pair of abdominal mammary glands (designated R4 and L4). Eight lactating mice were inoculated with 10^8 cfu of *S. aureus* in 0.1 ml saline in both R4 and L4. This was followed one hour later by the infusion of 10 ug lysostaphin in 0.1 ml saline into R4 and 0.1 ml saline into L4. After a further 30 minutes the mice were killed and the mammary glands were aseptically removed and homogenized in saline containing 0.1 mg/ml trypsin (Sigma Chem.) to destroy active lysostaphin. Ten fold dilutions were placed on 7 per cent calf blood agar (Oxoid Blood Agar Base Number 2), incubated at 37°C overnight and viable counts determined. In a fur-

ther experiment using 20 mice a prophylactic use of lysostaphin was simulated by infusing 10 ug of lysostaphin intramammarily, followed either immediately or after one hour by 10^3 cfu of *S. aureus*. Control glands were infused with saline instead of lysostaphin. After 24 hours the mice were killed and dissected. Gross pathological changes were noted an viable *S. aureus* counts determined as described above.

RESULTS

Infusion with 10mg lysostaphin into mammary glands previously inoculated with *S. aureus* reduced bacterial recoveries, compared to the controls, by more than 99 per cent in 30 min. This reduction was statistically significant ($t=2.56$; $P<0.02$). When 10 ug of lysostaphin was administered either immediately or one hour before *S. aureus* inoculation, recoveries after 24 hours averaged around 10^2 viable *S. aureus* per mammary gland compared with approximately 10^9 per mammary gland for the saline treated controls. In the latter case, the control glands showed severe pathological changes typical of acute staphylococcal mastitis in the mouse. The control glands were darker and reddened, had a brittle texture and some areas of liquefaction and haemolysis. Histological sections revealed a severe inflammation, infiltration of neutrophils and macrophages with areas of coagulative necrosis. Large numbers of *Staphylococci* were visible. In contrast, the lysostaphin treated glands remained pale and elastic with only slight reddening around the base of the teat. Histological examination showed little or no cellular infiltration, a well preserved and functioning alveolar structure and few *cocci*.

Conclusion

These experiments clearly demonstrate the anti-staphylococcal activity of lysostaphin *in vivo*. Both a therapeutic and prophylactic potential were demonstrated. The cloning of the lysostaphin gene may make it readily available for therapeutic use at a competitive price and its relatively high specificity makes it attractive for use in food-producing animals. Furthermore, advances in transgenic technology allow the direction of the expression of transgenes to the mammary gland of ruminants (Simons et al., 1987, *Nature*, 328:530). In general, this has been applied to the production of pharmacologically active substances for use in human medicine. However, the incorporation and expression of the lysostaphin gene in the lactating mammary gland could potentially increase the resistance of the animal to staphylococcal mastitis.

EXAMPLE IV

Lysostaphin efficacy for treatment of *Staphylococcus aureus* intramammary infection

Cloned-derived lysostaphin was evaluated as to its bactericidal effect on *S. aureus* intramammary infections. *S. aureus* (Newbould 305) was eliminated from glands of guinea pigs 48 hrs post-infection by 125 µg of lysostaphin in 14/16, 25 µg in 5/8, 5 µg in 5/10, 1 µg in 0/1, and 0 µg in 0/3. Glands infected with *S. aureus* at 48 hours post-challenge in untreated guinea pigs persisted, however, 3/25 control glands of treated guinea pigs cleared in response to treatment of the adjacent gland.

Somatic cell/ml in guinea pig shifted from 10^4 pre-infected glands to cell counts greater than 3 x 10^6 following *S. aureus* inoculation. Treatment with lysostaphin caused a neutrophilic shift in the treated gland to levels exceeding 108 accompanied by an increase in the adjacent non-treated gland but dropped sharply to pre-treatment level. The greatest response in control glands was observed in animals receiving 125 ug which corresponded to 2/25 clearance of *S. aureus* in control glands.

The leukocyte response to intramammary treatment in the cow is similar to the guinea pig model described above. Somatic cell levels increased tenfold in *S. aureus* infected glands at the milking following treatment. Cell levels returned to pre-treatment levels or lower in subsequent milking. A rise in leukocytes alone could not account for clearance of the infection.

EXAMPLE V

Use of a recombinant bacterial enzyme (Lysostaphin) as a mastitis therapeutic

A recombinant mucolytic protein, lysostaphin, was evaluated as a potential intramammary therapeutic for *Staphylococcus aureus* mastitis in dairy cattle. Lysostaphin, a product of *Staphylococcus simulans*, enzymatically degrades the cell wall of Straphylococcus *aureus* and is bactericidal.

Thirty Holstein-Friesian dairy cattle in their first lactation were infected with *Staphylococcus aureus* (Newbould 305, ATCC 29740) in all quarters. Infections were established and monitored for somatic cell counts and *Staphylococcus aureus* colony-forming units 3 weeks prior to subsequent treatment. Infected animals were injected through the teat canal with a single dose of recombinant lysostaphin (rLYS) (dose 1

to 500 mg) or after three successive p.m. milking with 100 mg of rLYS in 60 ml of sterile phosphate-buffered saline. Animals were considered cured if the milk remained free of *Staphylococcus aureus* for a total of 28 milkings after the last treatment.

RESULTS

Kinetic analysis of immunologically active rLYS demonstrated that a minimum bactericidal concentration was maintained in the milk for up to 72 hours at 37°C. In contrast, penicillin G retained less than 10% of its bacteriostatic activity over the same incubation time.

Dose titration and kinetics of rLYS in the bovine mammary gland

In order to determine the optimal effective dose to elicit long-term cures, a titration was performed in which a single dose of rLYS at concentrations of 0, 1, 10, 100, or 500 mg was administered. Untreated quarters and the 1-mg treatment failed to clear all quarters of *S. aureus*. The 10- 100- and 500-mg does transiently cleared the milk of *S. aureus* for at least one milking. In relapsed quarters, the length of time of the milk remained clear of *S. aureus* was approximately proportional to the dose administered. Fourteen days after treatment, two quarters were cured with the 100 mg dose and one with the 500 mg dose. Because rLYS maintains a minimal bactericidal concentration (MBC) for approximately 24 h and the experimental infections undergo a 2- to 4- days cycling, multiple infusions of 100 mg of rLYS over three consecutive milking were determined to be optimal to maintain a minimal effective dose for 3 to 5 days and to elicit cures.

Conclusion

Staphylococcus aureus is one of the primary etiologic agents of bovine mastitis and a major cause of economic loss to the dairy industry. An effective mastitis therapy for the lactating dairy cow remains a major unfilled need. Because current therapy is only moderately efficacious and is costly because of milk discard and culling infected animals, treatment only during the dry period has been the adopted herd management practice of choice. Neither approach addresses the majority of the infections in a lactating animal, which are chronic and subclinical in nature. A recombinant protein such as rLYS with bactericidal activity against *S. aureus* could be an extremely useful therapeutic to the veterinarian. If rLYS was as efficacious as antibiotics, natural proteolysis and inactivation in the milk of rLYS, as well as inactivation during ingestion by the consumer, would potentially minimize any concerns associated with residues in milk.

The *in vivo* does titration suggested that the minimal effective therapeutic dose was 100 mg of rLYS. However, therapeutically, it would be desirable to administer multiple infusions of rLYS to maintain a minimal bactericidal activity within the milk of treated glands for one to three successive milkings. The *in vivo* bactericidal activity of rLYS was most effectively demonstrated by the fact that 95% of the quarters cleared the milk of detectable *S. aureus* for a minimum of one milking after the last intramammary infusion.

EXAMPLE VI

Expression of jet-injected plasmid DNA in the ovine mammary gland

A jet-injection based DNA delivery system has been evaluated as a means to transiently transfect the

lactating mammary gland *in vivo* and as a technique for DNA vaccination. The model expression plasmid contained the human growth hormone (hGH) gene driven by the human cytomegalovirus immediate early gene 1 promoter/enhancer region (CMV). Expression from the naked plasmid DNA jet-injector into lactating mammary glands of sheep was sufficient to be detected by Northern blot analysis when tissue was obtained 48 hours after *in vivo* transfection. In conclusion, the ability to transiently transfect lactating mammary tissue *in vivo* circumvents the difficulties encountered with *in vivo* culture techniques and provides a method for examining mammary regulatory elements and testing of fusion gene constructs designed for the production of transgenic animal bioreactors.

EXAMPLE VII

Elimination of *Staphylococcus aureus* in an eukaryotic system expressing the lysostaphin

The lysostaphin gene was introduced into 293 cells (human fetal kidney cells) maintained *in vitro*. The recombinant bacteriocin, the lysostaphin, was secreted in the medium culture and was found to kill contaminant *S. aureus* during the challenge.

The lysostaphin gene was obtained by PCR amplification from extracted DNA of *Staphylococcus simulans* biovar *staphylolyticus* (NRRL B-2628), and *Staphylococcus aureus* strain Newbould (ATCC) was used for the challenge in transfected eukaryotic cells. Staphylococcal strains were grown in Brain Heart Infusion (BHI) medium.

Purification of the lysostaphin gene

Staphylococcus simulans biovar *staphylolyticus* was cultured overnight in a stirring incubator at 37°C. The media was centrifuged, and the pellet was resus-

pended in 5 ml of 50 mM EDTA-50mM Tris-HCL (pH 7.8) containing 50 mg of lysostaphin (Sigma) ml^{-1} and the suspension was incubated at 37°C for 2 hours. Purified bacterial DNA was directly amplified by PCR method to isolated the lysostaphin gene. The set of oligonucleotide primers used were as followed: 5'-TTAAGGTTGAAGAAAACAATT-3' (SEQ ID NO:1) and 5'-GCGCTCACTTTATAGTTCCCCAA-3' (SEQ ID NO:2). The amplification was performed by using a Thermal DNA cycler and 2.5 units of Taq DNA polymerase (Perkin Elmer Cetus), and a 30 cycles program with an annealing step at 60°C for 30 sec., elongation at 72°C for 90 sec. and denaturation at 93°C for 10 sec. The PCR product was composed by the entire lysostaphin sequence, including the coding gene with the aminoterminal pre- and pro- regions. All other recombinant DNA procedures, including restriction endonuclease digestion, ligation, washing with phenol-chloroform mixture, ethanol precipitation, transformation and cloning of the constructs in *E. coli* strain DH5a, were carried out by standard methods. All enzymes were from Boehringer Mannheim.

The lysostaphin was linked to an eukaryotic expression vector including the human cytomegalovirus immediate early gene 1 promoter/enhancer region (CMV) and the human interleukin-2 signal peptide.

Cell culture and DNA transfection

293 cells, a human foetal kidney cell line transformed by an origin-defective mutant of simian virus 40, were cultured in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% (vol/vol) fetal calf serum (Gibco BRL) and glutamine (1.4 mM). The cells were seeded into 30-mm wells at 500 000 cells par well and grown in 2 ml of medium for 24h at 37°C (in air atmosphere containing 5% CO_2) to yield 50 to 60%

introduced into the cells by the calcium phosphate method with the following modifications. The precipitate containing 7.5 µg of DNA was added to 2 ml of culture medium. After 24 h, the medium was replaced with 2 ml of medium per well, and samples of the medium were harvested at each 24 h following transfection to evaluate the production of the lysostaphin by Western blot analysis and ELISA

Assay for biological active lysostaphin

The wells containing the transfected 293 cells were infected with 10^2 or 10^3 of *Staphylococcus aureus* Newbould. Samples of 100µl of the infected medium were spreaded on sheep blood agar. After incubation for 24 h at 37°C, the number of colony forming units (CFU) was evaluated to assess the inhibition effect of the recombinant lysostaphin on the growth of the bacteria.

RESULTS

Production of recombinant lysostaphin by transfected eukaryotic cells

The modified lysostaphin gene was transfected into tissue culture cells to demonstrate the expression, processing and activity of the enzyme on infecting bacteria. After analysis of the culture medium, a band of approximately 25 kDa was generated; this band was similar in size to mature lysostaphin. The same result was observed in other experiments in which the expression of recombinant lysostaphin has been carried out in eukaryotic cells. The ELISA assays have revealed that the recombinant lysostaphin was produced in concentrations of 100 to 250 ng/ml/24h depending of the clone.

Activity of the lysostaphin secreted by mammalian cells

The activity of the recombinant lysostaphin secreted by transfected mammalian cells has been observed by its efficiency to reduce or in some replicates to inhibit the growth of infecting *Staphylococcus aureus* in the culture media. Samples of media taken from non-transfected cells have shown none inhibitory effect on the development of the bacteria present in the wells. The plates of agar were completely confluent after overnight incubation. In contrast, when an initial amount of 10^3 bacteria was cultured in presence of transfected eukaryotic cells, very few CFU were counted on the plates. Less than 100 CFU were observed in our assays when 10^3 bacteria were used, while we did not observed the presence of CFU on gels when 10^2 bacteria were added to the wells containing the transfected cells.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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(ii) TITLE OF INVENTION: ANIMAL GENE THERAPY

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9509461.1
(B) FILING DATE: 10-MAY-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTAAGGTTGA AGAAAACAAT T 21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCGCTCACTT TATAGTTCCC CAA 23